Advances in Natural and Applied Sciences, 2(3): 178-184, 2008
ISSN 1995-0772

ORIGINAL ARTICLE

Ethylene Inhibitors Promote *in vitro* Regeneration of Medium Staple Cotton (*Gossypium hirsutum* L.) Cultivar Barac B- 67

Eltayb Abdellatef and M.M. Khalafalla

Commission for Biotechnology and Genetic Engineering, National Centre for Research, Khartoum, Sudan

Eltayb Abdellatef and M.M. Khalafalla, Ethylene Inhibitors Promote *in vitro* Regeneration of Medium Staple Cotton (*Gossypium hirsutum* L.) Cultivar Barac B- 67, *Adv. in Nat. Appl. Sci., 2(3): 178-184, 2008*

ABSTRACT

The promotive effects of ethylene inhibitors, silver nitrate ($AgNO_3$) and cobalt chloride ($CoCl_2$) on *in vitro* shoot regeneration from 35-day-old seedling-derived cotyledonary node explant of medium staple cotton (*Gossypium hirsutum* L. cv Barac B- 67) was investigated. Shoot development was induced on cotyledonary node devoid of cotyledons and apical meristems cultured on B5 medium supplemented with kinetin (Kn) or benzyladenine (BA) alone or in combination. Both types and concentrations of cytokinins significantly influenced shoot proliferation. Kn proved to be more effective than BA. The efficiency of both BA and Kn for multiple shoot induction was positively affected when used in combination. The best response, however was obtained when 2.5 mg/L Kn was used in combination with BA 0.1 mg/L Addition of silver nitrate $AgNO_3$ and Cobalt chloride $CoCl_2$ to the medium enhanced regeneration frequency as well as number of shoots obtained per explant. The best result (3.2 shoot/explant) was obtained by using $AgNO_3$ at 3.0 mg/L. Elongation of multiple shoots was obtained on half strength agar-solidified B5 basal medium without plant growth regulators. More than 87% of the *in vitro* induced shoots produced roots when cultured on half strength B5 medium supplemented with 2.0% sucrose, 0.8% agar and 0.1 mg /L Naphthalene Acetic Acid (NAA). Rooted plants were hardened and 95% survived under greenhouse conditions.

Key words: *In vitro* regeneration, cotton, silver nitrate, cobalt chloride. *G. hirsutum*

Introduction

Decades of traditional breeding have been used in the improvement of agronomic traits of cotton (*Gossypium*). However, there remains of useful economic characters, including pest and disease resistance, stresses tolerance and that may be amenable for further genetic enhancement of commercial cotton cultivars using modern breeding methods based on gene transfer technology.

The limiting step to the successful use of modern techniques in genetic improvement of the major crops has not been transgene insertion itself, but rather the regeneration of viable plants from the transgenic explant material (Murphy, 2003). Therefore, the success of any cotton improvement programme using gene transfer technological tools such as biolistics and *Agrobacterium*-mediated transformation depends on the availability of plant regeneration systems that are genotype-independent, efficient and which do not yield somaclonal variant (Firoozabady *et al*., 1987; Gould and Cedeno, 1998; McCabe and Mattinell, 1993).

The composition of the culture medium and the gaseous environment are important factors in regeneration of shoots *in vitro*. In recent years there has been increasing evidence that the occurrence of morphogenesis in cultured plant cells may be associated with ethylene (Saha *et al*., 2007).Varying amounts of ethylene is released in culture vessels during *in vitro* regeneration (De Proft *et al*., 1985). However, the role of ethylene in plant cells and tissues grown *in vitro* is not well understood. The presence of ethylene was found to be important for shoot morphogenesis in rice callus (Adkins *et al*., 1990) embryogenesis from anther cultures of *Hordeum vulgaris* (Cho and Kasha, 1989)

Corresponding Author: M.M. Khalafalla, Commission for Biotechnology and Genetic Engineering, National Centre for Research, P.O. Box 2404. Khartoum, Sudan.
Mobile: 00249-122861713 E-Mail: Mutasim@myway.com mkhalafalla@ncr.sd

and flower bud formation from thin-layer explants of *Nicotiana tabacum* (Smulders *et al*., 1990). In contrast, ethylene accumulation inhibits *in vitro* shoot regeneration in *Nicotiana* (Huxter *et al*., 1981), *Triticum* (Purnhauser *et al*., 1987), *Zea mays* (Songstad *et al*., 1988) and *Brassica* (Chi *et al*., 1991). It was also observed that the addition of certain chemicals such as cobalt chloride or silver nitrate to the culture medium can inhibit ethylene production or its function by blocking certain steps in the pathway (Pua and Chi, 1993) and increases *in vitro* regeneration in monocots (Purnhauser *et al*., 1987; Songstad *et al*., 1988) and dicots (Chi et al *et al*., 1991; Roustan *et al*., 1989). Considering these information, the objective of this work was to describe the effect of silver nitrate and cobalt chloride on the frequency of adventitious shoot regeneration and number of shoots per explant of *in vitro* culture of cotyledonary node of medium staple cotton cultivar Barac B-67.

Material and methods

Plant material

Seeds of medium staple cotton cultivar (Barac B - 67) used in this study were obtained from the Agricultural Research and Technology Corporation (ARTC), Wad Medani, Sudan

Seeds delinting and surface sterilization

Seeds were delinted by using concentrated commercial H_2SO_4 (100 ml/kg of seeds). The seeds were continuously stirred in H_2SO_4 by spatula for 1 minute then washed by continuously running tap water for another 1 minute followed by thorough washing in sterile distilled water to remove traces of surface adherent. Under laminar flow cabinet seeds were disinfected by soaking in mercuric chloride $HgCl_2$ 0.2% (w/v) for 15 mints with continuous shaking and finally washed for five times by sterilized distilled water.

Seed germination and explant preparation

After surface sterilization 100 seeds were transferred to culture bottle and directly inoculated on the B5 (Gamborg *et al*., 1968) basal media and incubated for germination at 25ºC±2 with a 16 h photoperiod. Cotyledonary nodes were removed from 35days-old *in vitro* raised seedlings. The cotyledons and apical meristem were excised and discarded. Thus, each explant had two dormant axillary buds. These decapitated cotyledonary nodes were used as explants throughout this study.

Effect of cytokinins on in vitro morphogenesis

Different concentrations (0.1, 0.5, 1.0, 2.5, or 5.0 mg/L) of BA and Kn alone or in combinations were added to the culture bottles containing B5 basal media in order to assess their effect on *In vitro* morphogenetic responses of the cotyledonary node.

Effect of ethylene inhibitors on in vitro morphogenesis

To determine the effects of ethylene inhibitors, B5 basal media containing Kn at 2.5 mg/L and BA 0.1 mg/L was supplemented with silver nitrate ($AgNO_3$) and cobalt chloride ($CoCl_2$) at different concentrations (1, 2, 3, 4, 5, 10 mg/L).

Rooting of in vitro produced shoots

Shoots (2-3 cm) derived from shoot bunches of cotyledonary node were excised and rooted on medium consisting of B5 and ½ B5 basal medium supplemented with 0.1,0.5,1.0 mg/L Naphthalene Acetic Acid (NAA) either Indole Acetic Acid (IAA) or Indole Butric Acid (IBA).

Culture condition and Data analysis

Cultures were incubated for six weeks at 25ºC±2 with a 16 h photoperiod. All the media used in this study were supplemented with 2% (w/v) sucrose, solidified with 0.8% (w/v) and the pH was adjusted to 5.5 before addition of the agar and autoclaving at 121°C and 15 lb psi for 15 min.

Results were observed at regular intervals and data were collected from three independent experiments and presented as average ± standard error (SE).

Results and discussion

Cotton seeds obtained from the field are highly contaminated as they are covered with large numbers of small hairs that can hold spores of fungi and bacteria. Therefore in this study cotton seeds were firstly delinted by using concentrated H_2SO_4 then sterilized by $HgCl_2$ before *in vitro* germination. Delinting with H_2SO_4 is a highly effective way to remove the hairs and reduce the risk of contamination in the cultures. Disinfection of seeds through delinting with concentrated H_2SO_4 and then followed by $HgCl_2$ has already been proved to be essential in cotton tissue culture (Abdellatef and Khalafalla, 2007; Abdellatef and Khalafalla, 2008; Rauf *et al*., 2004).

Direct shoot bud differentiation was observed after 2 weeks of culture initiation. Multiple shoots were initiated from the cotyledonary node explants after 4 weeks of culture (Fig.1b). The frequency of shoot formation was influenced by types and concentrations of cytokinins used (Table1). Explants obtained from 35-days old seedling cultured on hormone-free B5 basal medium failed to show any response but remained green up to four weeks. However, on B5 basal medium supplemented with various concentrations of BA or Kn alone or in combinations enlarged in their size after one to two weeks of culture and adventitious shoots developed directly in another four weeks (Table1). These results agree with the finding of Jorge *et al*., (1998) who found that cytokinin is directly responsible for reprogramming the embryonic apical meristem axes of cotton towards the multiplication of buds.

Generally determination of optimal concentrations and combinations of growth regulators is necessary because unfavorable concentration may inhibit the growth of cellular mass as reported by Moore, (1998). Furthermore, the dose of cytokinin is known to be critical in multiple shoots induction (Abdellatef and Khalafalla, 2007). Therefore, in this study we compared the effects of various concentrations of BA and Kn on multiple shoots induced on cotyledonary node explants. The result showed that Kn at different concentrations induced more shoots per explant compared to BA at the same concentrations (Table 1). Indicating that, Kn was more efficient than BA for multiple shoot production from cotton cotyledonary node explant. Similar result was reported by Abdellatef and Khalafalla, 2007. The shoot regeneration frequency increased with increases of Kn concentration until it reaches 2.5 mg/L, which was found to be the optimal concentration for maximum frequency of shoot bud formation (2.6 shoots per explant). However, at higher concentration of both cytokinins the number of shoot per explant was reduced (Table 1). Zapata *et al*., (1999) had a similar observation with cotton shoot apices where plant regeneration was suppressed with higher concentrations of BA. This is mainly, because at higher cytokinin level cotyledonary node explant produced excessive callus and failed to improve the efficiency of shoot multiplication. Thiem, (2003) reported that callus growth on explant usually interfere with the propagation process.

Table 1: Effects of cytokinin and cytokinin combination on multiple shoots induction on cotyledonary node explants obtained from 35-day-old seedlings of medium staple cotton cultivar (Barac- B- 67)

Cytokinin (mg/L)			
BA	Kin	Reg. culture (%)	No shoot/ explant (mean ± SE)
0.1	-	100	2.0±0.0
0.5	-	100	2.0±0.2
1.0	-	100	2.4±0.1
2.5	-	100	2.0±0.0
5.0	-	0	0.0±0.0
-	0.1	100	2.1±0.1
-	0.5	100	2.3±0.0
-	1.0	100	2.4±0.1
-	2.5	100	2.6±0.1
-	5.0	75	1.8±0.1
0.1	0.1	100	2.0±0.1
	0.5	100	2.2±0.0
	1.0	100	2.2±0.1
	2.5	100	2.8±0.1
	5.0	100	2.7±0.1
0.5	0.1	100	1.6±0.1
	0.5	100	2.0±0.0
	1.0	100	2.0±0.1
	2.5	100	2.4±0.1
	5.0	100	1.8±0.1
1.0	0.1	100	2.3±0.1
	0.5	100	2.0±0.0
	1.0	25	2.3±0.1
	2.5	16.6	2.3±0.2
	5.0	0	0.0±0.0
2.5	0.1	100	1.8±0.1
	0.5	100	0.5±0.2
	1.0	100	0.3±0.2
	2.5	0	0.0±0.0
	5.0	0	0.0±0.0

Table 2: Effect of ethylene inhibitors (silver nitrate and cobalt chloride) on multiple shoots induction on cotyledonary node explants of medium staple cotton cultivar (Barac- B -67) cultured on shoot formation media

Ethylene inhibitor (mg/L)			
$AgNo_3$	$CoCl_2$	Regeneration %	No of shoot/Explant (Mean ± SE)
1.0	0.0	100	2.5±0.2
2.0	0.0	100	2.7±0.2
3.0	0.0	100	3.2±0.1
4.0	0.0	100	2.6±0.2
5.0	0.0	100	2.4±0.2
10.0	0.0	100	2.3±0.1
0.0	1.0	100	2.5±0.2
0.0	2.0	100	2.9±0.1
0.0	3.0	100	3.1±0.1
0.0	4.0	100	2.8±0.0
0.0	5.0	100	2.6±0.0
0.0	10.0	100	2.0±0.0

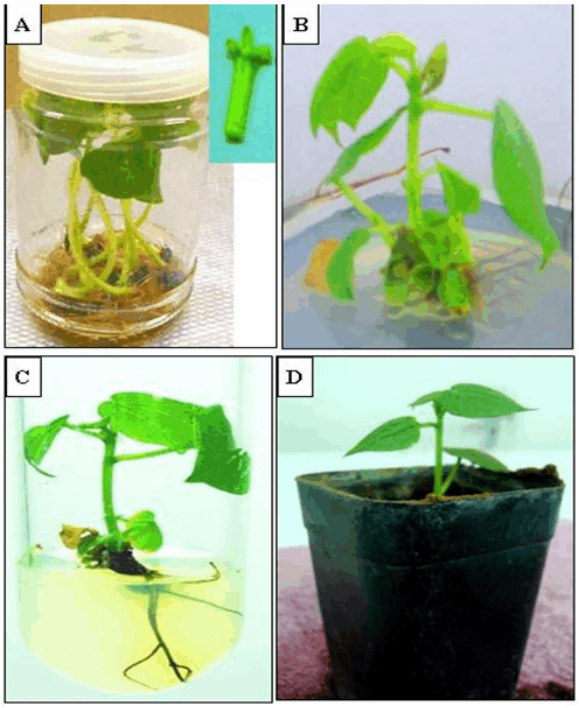

Fig. 1*:* *In vitro* induction of multiple shoots and plant regeneration of medium staple cotton cultivar (Barac-B-67) A. *In vitro* germinated seedling and cotyledonary node explant obtained from 35 days - old – seedling. B. Multiple shoots bunches induced from cotyledonary node explants on shoot formation media supplemented with $AgNo_3$ 3.0 mg/L. C. *In vitro* rooted shoot on half -strength B5 basal medium supplemented with NAA (0.1 mg/L). D. *in vitro* regenerated cotton plants in established soil.

In this study the result showed that combinations of Kn and BA positively affected the multiplication rate of the *in vitro* induced shoot compared to that obtained with cytokinine alone (Table 1). Kn 2.5 mg/L in combination with BA 0.1 mg/L induced the maximum No of shoot (2.8 shoot/ explant). The synergistic action of a combination of two or more cytokinins has also been reported in cotyledonary nodes of *Eclipta alba*, (Baskaran and Jayabalan, 2005). In *E. impensa*, shoot segments incubated on a medium supplemented with 0.25mM BAP and 2.5mM kinetin produced more healthy shoots and overall shoot quality was better than observed for BAP used alone (Bunn, 2005). However, the multiple shoots obtained on combination of cytokinins failed to elongate on the same medium. Based on previous study carried in our laboratory (in press), it was found that half strength B5 basal medium without plant growth regulators was the most suitable media for shoot elongation, therefore for elongation shoot produced on B5 basal medium supplemented with Kn 2.5 mg/L and BA 0.1 mg/L were transferred individually to culture bottles containing half strength B5 basal medium without plant growth regulators and supplemented with 2% sucrose and 0.8% agar for 15 – 20 days. Cytokinin has been reported to regenerate cotton plants with short and compact shoots (Banerjee, 2000). Moreover, as in this study, cytokinins have often been reported to stimulate shoot proliferation, while inhibiting shoot elongation (Brassard, 1996). The use of hormone-free medium for shoot elongation has already been reported for soybean (Kaneda *et al*., 1997) and faba bean (Khalafalla and Hattori, 1999).

Table 3: The Effect of auxin and basal media strength on rooting of *In vitro* derived shoots of medium staple cotton cultivar (Barac B-76) after 6 weeks of culture

Auxin (mg/L)			Rooting %		No of roots/shoot (Mean ± SE)	
NAA	IAA	IBA	½ B5	Full B5	½ B5	Full B5
0.0	0.0	0.0	37.0	50	0.8±0.3	0.6±0.3
0.1	0.0	0.0	87.0	75	1.4±0.3	0.8±0.3
0.5	0.0	0.0	75.0	50	0.8±0.2	0.9±0.2
1.0	0.0	0.0	50.0	50	1.1±0.5	1.0±0.5
0.0	0.1	0.0	37.0	37	0.9±0.5	0.9±0.4
0.0	0.5	0.0	50.0	50	0.5±0.2	1.0±0.2
0.0	1.0	0.0	62.5	50	1.1±0.4	1.1±0.4
0.0	0.0	0.1	50.0	50	0.4±0.2	0.5±0.2
0.0	0.0	0.5	50.0	50	0.8±0.3	1.0±0.3
0.0	0.0	1.0	62.5	75	0.8±0.3	1.1±0.3

Cytokinins are ethylene inducing plant hormones and known to increase ethylene production several folds in many plants (Saha *et al*., 2007), at least partially through the increase in ACC synthase activity (Ables *et al*., 1992). BA synergistically enhanced ethylene production in mungbean hypocotyls (Yoshi and Imaseki, 1982). Kn slightly stimulated ethylene production by etiolated seedlings in several species including pea and mung bean (Saha *et al*., 2007). Based on the above-mentioned evidences the effects of ethylene inhibitors on cotton multiple shoot induction was addressed by adding different levels of silver nitrate and cobalt chloride to the culture media containing Kn at 2.5 mg/L and BA 0.1 mg/L. Our result showed that, the presence of ethylene inhibitors namely $AgNO_3$ (3.0 mg/L) or $CoCl_2$ (1.0 mg/L) in the shoot regeneration medium (B5 0.1 mg/L BA, ??2.5 Kn mg/L) was found to be beneficial as they significantly enhanced the percentage shoot regeneration and number of regenerated shoots per explant (Fig.1b) (Table 2).The interaction of ethylene with other plant growth regulators is highly complex and is still little understood. Interestingly, there are many contrasting examples which show that the regulation of ethylene levels in tissue cultures can have both positive and negative effects on *in vitro* morphogenesis and proliferative growth. Ethylene was shown to inhibit shoot regeneration in callus cultures of sunflower and tobacco (Huxter *et al*., 1981). The negative effect of ethylene on morphogenesis in maize callus culture was demonstrated (Songstad *et al*., 1988). Moreover, as reported in several recalcitrant plant species such as chinese cabbage (Chi *et al*., 1991), mustard (Pua and Chi, 1993), rice (Adkins *et al*., 1993). On the other hand, positive morphogenetic response of ethylene was reported in a few tree species. In Norway spruce it enhanced the induction of embryogenic tissues (Kvaalen, 1994), whereas in *Pinus radiata*, it promoted shoot bud differentiation in cotyledon explants (Kumar *et al*., 1987) and in eastern white cedar, axillary shoot elongation (Nour and Thorpe, 1994). The above contrasting results show that the role of ethylene in *in vitro* morphogenesis, perhaps, vary from species to species and thus needs to be examined with each plant genotype. Keeping this in view, in the present study the effect of two ethylene inhibitors $AgNO_3$ and $CoCl_2$ on adventitious shoot proliferation from cotyledon node was investigated. $AgNO_3$ is believed to inhibit ethylene action by competing with ethylene for binding sites located predominantly at the intracellular membrane (Beyer, 1976,). In the present study, although both the ethylene inhibitors promoted adventitious shoot regeneration from cotyledon explants, $AgNO_3$ appeared to be more effective than $CoCl_2$. The stimulation of shoot morphogenesis elicited by $AgNO_3$ or $CoCl_2$ is in agreement with other reports on *Brassica campestris* (Chi and Pua, 1989), *Capsicum annuum* (Hyde and Philips, 1996), *Manihot esculenta* (Zhang *et al*., 2003), *Triticum aestivum* and *Nicotiana plumbaginifolia* (Purnhauser *et al*., 1987).

For *In vitro* rooting of the regenerated shoots, half strength B5 basal medium was found to be more effective than full strength. Also NAA gave better result for root induction compared to basal media without or with IAA or IBA. Among the different concentrations of NAA, rooting of cotton shoots was higher (87%) on half-strength B5 medium containing 0.1 mg/L NAA (Table 3) (Fig.1c). The promotory effect of reducing the salt concentration of basal medium and using of NAA on rooting of *in vitro* induced shoots was reported for cotton (Abdellatef and Khalafalla, 2007; Agrawal *et al*., 1997).

For acclimatization, plant were removed from rooting medium and transferred to plastic pots containing autoclaved soil and covered with glass bottle to maintain humidity and were kept under culture room conditions for one week. After three weeks, glass bottles were removed and transferred to green house and placed under shade until growth was observed, 95% of the plants survived and all were morphologically normal (Fig. 1d).

Conclusion

Development of an efficient tissue culture and plant regeneration protocol for elite Sudanese cotton cultivars is the first step towards the application of transgenic technology to improve cotton breeding and is, thus, the foundation of cotton biotechnology. Furthermore, the present finding of enhancement of multiple shoot induction by the addition of various additives will promote the application of plant tissue culture technology in the area of selection resistance and production of cotton artificial seeds.

Acknowledgment

We gratefully acknowledge the financial support of the National Center for Research, Ministry of Science and Technology, Sudan. We wish to thank Professor Abdelbagi Mukhtar Ali, Agricultural Research and Technology Corporation (ARTC), Wadmadani station, for providing us with the seeds materials.

References

Abdellatef, E. and M.M. khalafallah, 2007. Adventitious shoot formation and plant regeneration in medium

Abdellatef, E. and M.M. Khalafallah, 2008. Influence of growth regulators on callus induction from hypocotyls of medium staple cotton *(Gossypium hirsutum L).* Cultivar Barac B -67. J. Soil. Nature, 2(1): 17-22.

Abeles, F.B., P.W. Morgan and ME. Jr. Saltveit, 1992. Ethylene in Plant Biology, Ed 2. Academic Press, New

Adkins, D.O., T. Shiraishi and J.A. McComb, 1990. Rice callus physiology-Identification of volatile emissions

Agrawal, D.C., A.K. Banerjee, R.R. Kolala, A.B. Dhage, A.V. Kulkarni, S.M. Nalawade, S. Hazra and K.V. Krishnamurthy, 1997. *In vitro* induction of multiple shoots and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant. Cell. Rep., 16: 647-652. and their effects on culture growth. Physiol. Plant, 78: 526-531. Appl. Biotech. Food. Sci. Policy, 1: 25-38.

Banerjee, A.K., D.C. Agrawal, S.M. Nalawade and K.V. Krishnamurthy, 2000. Recovery of *in vitro* cotton shoots through micro grafting. Curr. Sci. India. 78(5): 623-626.

Baskaran, P., N. Jayabalan, 2005. An efficient micropropagation system for Eclipta alba – a valuable medicinal herb. In Vitro Cell. Dev. Biol. Plant, 41: 532-538.

Beyer, E.M. Jr, 1976. A potent inhibitor of ethylene action in plants. Plant Physiol, 58: 268-271.

Brassard, N., L. Brissette, D. Lord and S. Laliberte, 1996. Elongation, rooting and acclimatization of micropropagated shoots from mature material of hybrid larch. Plant. Cell. Tiss. Org., 44: 37–44.

***Brassica campestris pekinensis*)Lour(Olsson *in vitro*. Plant.Physiol. 96: 178-183.**

Cho, U.H. and K.J. Kasa, 1989. Ethylene production and embryogenesis from barley anthers. Plant. Cell. Rep. 8:

Bunn, E. Development of in vitro methods for ex situ conservation of Eucalyptus impensa, an endangered mallee from southwest Western Australia. Plant Cell Tiss. Organ Cult. 83:97–102; 2005.

Chi, G.L. and E.C. Pua, 1989. Ethylene inhibitors enhanced *de novo* shoot regeneration from cotyledons of *Brassica campestris* spp. *in vitro.* Plant Sci., 64: 243-250.

Chi, G.L., E.C. Pua and C.J. Goh, 1991. Role of ethylene on de novo shoot regeneration cotyledonary explants of

DeProft, M.P., L.J. Maene, P.C. Debergeh, 1985. Carbondioxide and Ethylene evolution in the Culture atmosphere of Magnolia cultured *in vitro*. Plant. Physiol. 65: 375-379.

Firoozabady, E, D.L. DeBoer, D. J. Merlo, E. L. Halk, L. N. Amerson, K.E. Rashka and E. E. Murray, 1987. Transformation of cotton by Agrobacterium and regeneration of transgenic plants. Plant. Mole. Biol. 10: 105-116.
formation of ACC synthase by ethylene. Plant. Cell. Physiol. 23: 639-649.

Gamborg, O. L., R. A. Miller and K. Ojima, 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res. 50: 151- 158.

Gould, J. H. and M. Magallanes-Cedeno, 1998. Adaptation of shoot apex Agrobacterium inoculation and culture to cotton transformation. Plant. Mol. Biol. Rep. 16: 1-10.

Huxter, J.T., T.A. Thorpe and D.M. Reid, 1981. Shoot initiation in light and dark grown tobacco callus; the role of ethylene. Physiol. Plant. 53: 319–326.

Hyde, C., G. Phillips, 1996. Silver nitrate promotes shoot development and plant regeneration of Chile pepper (*Capsicum annum* L.) via organogenesis. In vitro Cell. Dev. Biol. Plant. 32:72-80.
in soybean (*Glycine max* (L.) Merr.). Plant. Cell. Rep. 17: 8–12.

Jorge, L. M., H. R. Permingeat, M. V. Romagnoli, C. M. Heisterborg and H.V. Ruben. 1998. Multiple shoot induction and plant regeneration from embryonic axes of cotton. Plant. Cell. Tiss. Org. 54: 131–13

Kaneda, Y., Y. Tabei, S. Nishimura, K. Harada, T. Akihama and K. Kitamura, 1997. Combination of thidiazuron and basal media with low salt concentrations increases the frequency of shoot organogenesis

Khalafalla, M. M. and K. Hattori, 2001. A combination of thidiazuron and benzyladenine promotes multiple shoot production from cotyledonary node explants of faba bean (*Vicia faba* L.). Plant. Growth. Regul. 27: 145–148.

Kumar, P.P., D.M. Reid and T.A. Thorpe.1987. The role of ethylene and carbon dioxide in differentiation of shoot buds in excised cotyledons of *Pinus radiata in vitro*. Physiol. Plant. 69: 244-252.

Kvaalen, H., 1994. Ethylene synthesis and growth in embryogenic tissue of Norway spruce: Effects of oxygen, 1-aminocyclopropane-1-carboxylic acid, benzyladenine and 2,4-dichlorophenoxyacetic acid. Physiol. Plant. 92: 109-117.
McCabe, D. E. and B. J. Martinell, 1993. Transformation of elite cotton cultivars via particle bombardment of meristems. Biotechnology. 11: 596-598.
Morre, J.l., HR. Permingeat, MV. Romagnoli, CM. Heisterborg, HR. Vallejos, 1998. Multiple shoot induction and plant regeneration fromembryonic axes of cotton. Plant Cell Tissue Organ cult. 54:131-136.
Murphy, D.J., 2003. Agricultural biotechnology and oil crops – current uncertainties and future potential.
Nour, K.A. and T.A. Thorpe, 1994. The effect of the gaseous state on bud induction and shoot multiplication *in vitro* in eastern white cedar. Physiol. Plant. 90: 163-172.
Pua, E.C. and G.L. Chi, 1993. *De novo* shoot morphogenesis and plant growth of mustard (*Brassica juncea*) *in vitro* in relation to ethylene. Physiol Plant 88: 467–474.
Purnhauser, L., P. Medgyesy, M. Czako, P.J. Dix and L. Marton, 1987. Stimulation of shoot regeneration in *Triticum aestivum* and *Nicotiana plumbaginifolia* viv. tissue cultures using ethylene inhibitor $AgN0_3$. Plant. Cell. Rep. 6: 1–4.
Rauf, S., M. Usman, B. Fatima, T. K. Manzoor and A. K. Iqrar, 2004. *In vitro* regeneration and multiple 415–417.

Roustan, J., A. Latche and J. Fallot, 1989. Stimulation of *Daucus carota* somatic embryogenesis by inhibitors of ethylene synthesis: Cobalt and nickel. Plant Cell Rep. 8 (3): 182-185.
Saha, S., H. Mori and K. Hattori, 2007. Synergistic Effect of Kinetin and Benzyl Adenine Plays a Vital Role in High Frequency Regeneration from Cotyledon Explants of Bottle Gourd (*Lagenaria siceraria*) in Relation to Ethylene Production, Breeding Science.57:197–202.
shoot induction in upland cotton)*Gossypium hirsutum* L.(. Int. J. Agri. Biol. 4: 704–707.
Smulders, M.J.M., A. Kemp, G.W.M. Barendse, F. Croes and G.J. Wullems, 1990. Role of ethylene in auxin-induced flower bud formation in tobacco explants. Physiol. Plant. 78: 167–172.
Songstad, D.D., D.R. Duncan and J.M. Widholm, 1988. Effect of 1-aminocyclopropane-1carboxylic acid, silver nitrate and non- bornadiene on plant regeneration from maize callus cultures. Plant. Cell. Rep.7: 262–265.
staple cotton)*Gossypium hirsitum* L.(cultivar)Barac B-67(. Int. J. Agri. Biol. 9)6(: 913- 916.
Thiem, B., 2003. *In vitro* propagation of isoflavone-producing *Pueraria lobata* (Willd.) Ohwi. Plant. Sci 165: 1123-1128.
Yoshi, H. and H. Imasekl, 1982. Regulation of auxin-induced ethylene biosynthesis: Repression of inductive
Zapata, C., S H. Park, KM. El- Zik and RH. Smith, 1999. Transformation of a Texas cotton cultivar by using Agrobacterium and the shoot apex. Theor. Appl. Genet. 98:252-256.
Zhang, P., J. Jaynes, I. Potrykus, W. Gruissem and J. Pounti-Kaerlas, 2003. Transfer and expression of an artificial storage protein)ASP1(gen in cassava)*Manihot esculenta Crantz*(. Trans. Res. 12:243-250.